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Abstract: **BACKGROUND:** Interferon- (IFN-) treatment suppresses HIV-1 viremia and reduces the size of the HIV-1 latent reservoir. Therefore, investigation of the molecular and immunologic effects of IFN- may provide insights that contribute to the development of novel prophylactic, therapeutic and curative strategies for HIV-1 infection. In this study, we hypothesized that microRNAs (miRNAs) contribute to the IFN- -mediated suppression of HIV-1. To inform the development of novel miRNA-based antiretroviral strategies, we investigated the effects of exogenous IFN- treatment on global miRNA expression profile, HIV-1 viremia, and potential regulatory networks between miRNAs and cell-intrinsic anti-HIV-1 host factors in vivo. **METHODS:** Global miRNA expression was examined in longitudinal PBMC samples obtained from seven HIV/HCV-coinfected, antiretroviral therapy-naïve individuals before, during, and after pegylated interferon- /ribavirin therapy (IFN- /RBV). We implemented novel hybrid computational-empirical approaches to characterize regulatory networks between miRNAs and anti-HIV-1 host restriction factors. **RESULTS:** miR-422a was the only miRNA significantly modulated by IFN- /RBV in vivo ($p < 0.0001$, paired t test; $FDR < 0.037$). Our interactome mapping revealed extensive regulatory involvement of miR-422a in p53-dependent apoptotic and pyroptotic pathways. Based on sequence homology and inverse expression relationships, 29 unique miRNAs may regulate anti-HIV-1 restriction factor expression in vivo. **CONCLUSIONS:** The specific reduction of miR-422a is associated with exogenous IFN- treatment, and likely contributes to the IFN- suppression of HIV-1 through the enhancement of anti-HIV-1 restriction factor expression and regulation of genes involved in programmed cell death. Moreover, our regulatory network analysis presents additional candidate miRNAs that may be targeted to enhance anti-HIV-1 restriction factor expression in vivo.

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Role of MicroRNA Modulation in the Interferon- α /Ribavirin Suppression of HIV-1 *In Vivo*

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Abstract

Background: Interferon- α (IFN- α) treatment suppresses HIV-1 viremia and reduces the size of the HIV-1 latent reservoir. Therefore, investigation of the molecular and immunologic effects of IFN- α may provide insights that contribute to the development of novel prophylactic, therapeutic and curative strategies for HIV-1 infection. In this study, we hypothesized that microRNAs (miRNAs) contribute to the IFN- α -mediated suppression of HIV-1. To inform the development of novel miRNA-based antiretroviral strategies, we investigated the effects of exogenous IFN- α treatment on global miRNA expression profile, HIV-1 viremia, and potential regulatory networks between miRNAs and cell-intrinsic anti-HIV-1 host factors *in vivo*.

Methods: Global miRNA expression was examined in longitudinal PBMC samples obtained from seven HIV/HCV-coinfected, antiretroviral therapy-naïve individuals before, during, and after pegylated interferon- α /ribavirin therapy (IFN- α /RBV). We implemented novel hybrid computational-empirical approaches to characterize regulatory networks between miRNAs and anti-HIV-1 host restriction factors.

Results: miR-422a was the only miRNA significantly modulated by IFN- α /RBV *in vivo* ($p < 0.0001$, paired t test; FDR < 0.037). Our interactome mapping revealed extensive regulatory involvement of miR-422a in p53-dependent apoptotic and pyroptotic pathways. Based on sequence homology and inverse expression relationships, 29 unique miRNAs may regulate anti-HIV-1 restriction factor expression *in vivo*.

Conclusions: The specific reduction of miR-422a is associated with exogenous IFN- α treatment, and likely contributes to the IFN- α suppression of HIV-1 through the enhancement of anti-HIV-1 restriction factor expression and regulation of genes involved in programmed cell death. Moreover, our regulatory network analysis presents additional candidate miRNAs that may be targeted to enhance anti-HIV-1 restriction factor expression *in vivo*.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

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Introduction

Induction of interferon- α (IFN- α) expression is a critical first step in the defense against a range of viral pathogens [1,2]. Several studies have demonstrated that IFN- α treatment potently suppresses HIV-1 viremia in chronically infected individuals [3–6]. A provocative recent report demonstrated that IFN- α treatment results in sustained viral suppression in the absence of antiretroviral therapy (ART) and significant reduction in the size of the HIV-1 reservoir in chronically-infected individuals [7,8]. A

related analysis of the effects of IFN- α /ribavirin therapy on the HIV-1 latent reservoir in HIV/HCV-coinfected individuals reported a similar, significant reduction in reservoir size [9]. These studies collectively demonstrate that IFN- α molecular pathways may be exploited to attack the HIV-1 latent reservoir and achieve HIV-1 eradication.

In contrast to exogenous IFN- α treatment, endogenous IFN- α production and associated gene expression patterns are curiously often associated with rapid HIV-1 disease progression, high viral

load and persistent inflammation rather than beneficial disease outcomes [10,11]. This paradox is mirrored in recent studies of LCMV infection suggesting that IFN- α is associated with both beneficial and detrimental disease outcomes, and disease progression is governed by the overall balance between the various, diverse effects of type I interferon [12,13]. Focused analyses of IFN- α molecular pathways *in vivo* may allow us to identify specific mechanisms underlying the beneficial effects of IFN- α treatment on the control and clearance of viral infection. IFN- α treatment has been previously associated with an increase in perforin and granzyme A expression by natural killer (NK) cells in HIV-1-infected individuals, suggesting that enhanced NK-mediated anti-HIV-1 cytolytic activity may contribute to viral suppression [14]. Our group recently published data suggesting that several host restriction factors including BST-2/tetherin and members of the tripartite motif (TRIM) and APOBEC3 families play critical roles in the interferon-mediated suppression of HIV-1 viremia in chronically-infected individuals [15,16] and in the control of HIV-1 *in vivo* in the absence of antiretroviral therapy (ART) [17]. In this study, we hypothesize that microRNAs (miRNAs) contribute to the IFN- α -mediated suppression of HIV-1 by repressing HIV-1 protein translation directly, or by regulating the gene expression of host factors affecting HIV-1 replication and persistence *in vivo*.

miRNAs are a class of small non-protein-coding RNAs (approximately 22 nucleotides in length) that pair with specific “target” messenger RNAs (mRNAs) and play a significant role in regulating gene expression by binding to mRNAs, thereby repressing translation or degrading the mRNA altogether [18]. Solitary miRNAs often regulate expression of multiple genes with related functions; therefore, changes in expression levels of a single miRNA can broadly affect a gene network and modify complex biological processes [18]. miRNAs play a pivotal role in many biological processes, including cellular differentiation and proliferation [19]. Aberrant miRNA levels are associated with a number of disease states including several types of cancer, in which miRNAs can act as tumor suppressors and oncogenes [20].

miRNAs of viral and host origin may influence host-virus interaction by acting as direct modulators of viral replication, as factors affecting viral susceptibility, and as indirect modulators of cellular genes that influence viral propagation [21–23]. In the context of HIV-1 infection, a main challenge is to determine the specific roles of the expanding inventory of human miRNAs in HIV-1 pathogenesis, including the functional consequences of miRNA-mRNA interactions [24]. Human miR-28, miR-125b, miR-150, miR-223, and miR-382 target the 3′ UTR of HIV-1 transcripts, interfering with HIV-1 accessory gene expression potentially shifting productive infection into latency in resting CD4+ T lymphocytes [25]. The difference in expression levels of several anti-HIV-1 miRNAs in monocytes and macrophages correlates with cellular permissibility to HIV-1 infection *in vitro* [26]. A recent report suggests that miR-148 regulates the expression of HLA-C at the host cell surface, and this regulatory activity is correlated with control of HIV-1 replication [27]. Taken together, these observations suggest that studying natural expression levels of pro- and anti-HIV-1 miRNAs may prove valuable in understanding susceptibility to infection, and miRNA manipulation may constitute a promising anti-HIV strategy in the future. Recent data suggest that type I interferon modulates cellular miRNA profile as an antiviral mechanism against hepatitis C virus [28]. The relevance of miRNA to the potent IFN- α -mediated suppression of HIV-1, however, remains to be addressed and is the focus of this study.

IFN- α monotherapy is not typically administered to HIV-1-monoinfected individuals. Combination therapy with pegylated IFN- α and ribavirin (IFN- α /RBV) is commonly used to treat HCV infection [4]. In this study, we analyzed longitudinal clinical specimens from IFN- α /RBV-treated, ART-naïve HIV/HCV-coinfected individuals to evaluate the role of miRNAs in the suppression of HIV-1 by IFN- α *in vivo*.

Methods

Subjects and specimen processing

Longitudinal samples were collected from seven HIV/HCV-coinfected individuals enrolled in the Swiss HIV Cohort Study ([SHCS], www.shcs.ch) [29] who underwent IFN- α /RBV treatment (Table S1). All subjects had PBMC samples available before, during and after IFN- α /RBV treatment (a post-treatment sample was not available for Subject A), were ART-naïve, and had detectable HIV-1 RNA at baseline. The same collection of samples was analyzed in two recent publications from our group that characterized the role of retroviral restriction factors in the IFN- α -mediated suppression of HIV-1 *in vivo* [15,16], and gene expression data from these prior studies were included in miRNA-mRNA network analyses presented in this report. The research was approved by the institutional review boards at each of the Swiss HIV Cohort Study sites where samples were collected: University Hospital Basel, University Hospital Bern, University Hospital Zurich, and Canton Hospital, St. Gallen. All human participants gave written informed consent.

Cellular microRNA expression profiling

Total RNA was extracted from PBMC using TRIzol reagent (Invitrogen). miRNA expression was determined by applying the Megaplex Pools protocol (Applied Biosystems). 300 ng of RNA from PBMCs of the seven patients (pre, during, and post time points) was reverse transcribed using the TaqMan MicroRNA reverse transcription kit in combination with the Megaplex RT Primers Pool A that allows the analysis of 377 human miRNAs which represent the most rigorously studied human miRNAs or Megaplex RT Primers Pool B that allows the analysis of 377 newly discovered human miRNAs and endogenous controls. The following cycling conditions were used: 40 cycles at 16°C for 2 minutes, 42°C for 1 minute and 50°C for 1 second followed by 1 step at 85°C for 5 minutes. Reverse transcription was followed by a preamplification of the miRNA cDNA target (2.5 μ L) using TaqMan PreAmp Master Mix kit and Megaplex PreAmp Primers Pool A or B (Applied Biosystems). The following cycling conditions were applied: denaturation for 10 minutes at 95°C, 1 step at 55°C for 2 minutes followed by 2 minutes at 72°C, and 12 cycles (95°C for 15 seconds, 60°C for 4 minutes). According to manufacturer’s instructions, the preamplified cDNA product was loaded onto TaqMan MicroRNA Array A or TaqMan MicroRNA Array B after mixing with water and TaqMan Universal PCR Master Mix, with Uracil-DNA glycosylase (UNG). The following real-time PCR protocol was used: 2 min at 50°C, 10 min at 95°C, 40 cycles of (30 s at 95°C and 1 min at 60°C). Real time PCR reactions were performed on an ABI ViA 7 Real-Time PCR System (Applied Biosystems). The results were analyzed using ABI ViA 7 Real-Time PCR software (Applied Biosystems), based on the comparative Ct method (delta deltaCt). The amplification signal was checked on each sample by ABI ViA 7 Real-Time PCR System software. Data were normalized using a modified global mean normalization strategy based on common targets. The global mean on common targets strategy calculates normalization factors based on the geometric mean of the Relative Quantities

(RQs) of all genes that are measured in all samples. It is a variant on the global mean normalization strategy and has proven to be the most accurate and sensitive approach to analyze high-throughput miRNA profiles [30,31]. Analyses were restricted to miRNAs that were detectable in a minimum of 80% of samples (i.e. miRNAs that were expressed in at least 16 out of the 20 samples analyzed in this study) [32]; 289 miRNAs were chosen for subsequent analysis based on this threshold criterion.

In-vitro analysis of IFN- α treatment effects

PBMCs were collected from eight healthy (HIV- and HCV-negative) donors. All donor samples are routinely tested for a comprehensive panel of bloodborne pathogens upon collection including HIV, HCV and HBV using ultrasensitive PCR (nucleic acid test yield) and serology. The healthy donor study protocols were approved by the UCSF Committee on Human Research. CD4+ T cells were isolated using bead-based negative selection (STEMCELL Technologies). Cells were plated at a million cells per well and treated with either 5 U/ml of IFN- α -A2 (R&D Systems) or media as a negative control. The expression of miR-422a was measured after 24 hours of stimulation using Taqman primers and probes (Life Technologies).

Quantitative PCR measurement of MLH1 and TP53 mRNA expression

RNA from PBMCs was transcribed into cDNA using the SuperScript VILO cDNA Synthesis Kit (Invitrogen). Quantitative real-time PCR measuring MLH1 and TP53 using Taqman real time PCR was performed using the ABI ViiA 7 Real-Time PCR System. Raw cycle threshold (Ct) numbers of amplified gene products were normalized to the housekeeping gene ribosomal protein, large, P0 (RPLP0) to control for cDNA input amounts. RPLP0 was chosen as the housekeeping gene based on our previous analyses of the same set of samples [16]. We previously tested a panel of six housekeeping genes (GAPDH, 18S, ACTB, PPIA, RPLP0, and UBC). The GeNorm algorithm [33] identified RPLP0 as the most stably expressed housekeeping gene. Fold induction was determined using the comparative Ct method [33].

Statistical analysis

We identified differentially expressed miRNAs between pre-, during, and post-IFN- α /RBV time points using paired t-tests for each miRNA. To adjust for multiple comparisons, false discovery rates (FDR) were computed using the Benjamini-Hochberg procedure [34]. Viral load values were \log_{10} transformed, and miRNA values were global-normalized and then \log_{10} transformed. The missing values for each miRNA were imputed by the minimum detected value minus 0.5. After \log_{10} transformation and imputation, the within-group standard deviations (median across microRNAs) were 0.79 for pre-IFN- α /RBV timepoints, and 0.88 for during-IFN- α /RBV timepoints.

miRNA interactome characterization

Lists of restriction factors and miRNAs that were modulated by exogenous IFN- α /RBV treatment were uploaded to the Ingenuity Pathway Analysis (IPA) tool (Ingenuity Systems, www.ingenuity.com), and were analyzed based on the IPA library of canonical pathways. IPA was implemented to create a genetic interaction network depicting known experimentally validated relationships.

miRNA-mRNA network inference

We used two variables to generate a network between miRNAs and anti-HIV-1 restriction factor mRNAs: 1) inverse expression

relationships between a given miRNA-mRNA pair, and 2) significant sequence homology between a given miRNA seed region and a restriction factor 3' UTR. miRNA-mRNA inverse expression relationships were determined using the Pearson correlation coefficient (p -value < 0.05 , $\rho \leq 0.07$). miRNA-mRNA sequence homology was determined by using blastn (<http://blast.ncbi.nlm.nih.gov/>) with word size 4, alignment length ≥ 5 , and no mismatches allowed. We required reverse-complementing matches between miRNAs and mRNAs, with E-value cutoff ≤ 1 .

Results and Discussion

We examined the effects of exogenous IFN- α treatment on PBMC miRNA profile, focusing on seven subjects before, during and after IFN- α /RBV therapy (Table S1). A total of 754 established miRNA targets were surveyed in PBMCs. Based on our threshold criterion (detectable expression in a minimum of 80% of samples), 289 miRNAs were chosen for subsequent analysis. We aimed to identify particular miRNA variants that were up- or down-regulated in PBMCs during IFN- α /RBV treatment consistently across individuals. IFN- α /RBV did not significantly affect expression levels of miRNA machinery genes (Fig. S1) suggesting that observed effects of IFN- α /RBV on particular miRNAs were specific in nature rather than the consequence of nonspecific shifts in global miRNA production. Of all 754 miRNAs measured, 45 miRNAs were significantly modulated by IFN- α /RBV *in vivo*, based on a paired t test and an uncorrected significance cutoff of $p < 0.05$ (Fig. 1, Table S2). Twelve miRNAs in this list have been previously associated with HIV-1 infection (Table S2). However, due to the large number of miRNAs surveyed, accounting for multiple comparisons is imperative. After correcting for false discovery rate to account for multiple comparisons, only one miRNA out of the 45 initially identified, miR-422a, was significantly modulated by IFN- α /RBV *in vivo* using our highly stringent statistical criteria ($p < 0.0001$, paired t test; FDR < 0.037) (Fig. 2A). We then performed a controlled *in vitro* experiment in the absence of ribavirin co-administration and HIV or HCV infection to determine if IFN- α treatment suppresses miR-422a expression in CD4+ T cells, the principal target cells of HIV-1 infection in peripheral tissues. This experiment was conducted to evaluate the possibility that miR-422a modulation by IFN- α occurs within the target cell and may therefore play a role in determining cell-intrinsic susceptibility to HIV-1 infection. CD4+ T cells were negatively selected from fresh peripheral blood collected from eight HIV- and HCV-uninfected donors and treated with IFN- α *in vitro*. IFN- α treatment significantly suppressed miR-422a expression in CD4+ T cells *in vitro* ($p = 0.016$), extending the primary result of our *in vivo* expression profiling experiment (Fig. 2B).

We next sought to determine if any miRNAs exhibited significant correlations with HIV-1 viral load in our study population. Results are presented in Table 1. Several of the miRNAs exhibiting correlations with plasma viremia have been previously associated with HIV-1. The copy numbers of seven miRNAs were significantly correlated with baseline, pre-IFN- α /RBV HIV-1 viral load ($p < 0.05$, Spearman's rank): miR-29a, miR-101, miR-195, miR-25#, miR-491, miR-503, and miR-885. The copy numbers of six miRNAs were correlated with HIV-1 viral load during IFN- α /RBV: miR-138, miR-Let-7e, miR-10a, miR-145#, miR-31, and miR-589. Lastly, log HIV-1 viral load reduction during IFN- α /RBV therapy was significantly correlated with fold change of the following four miRNAs: miR-30e-3p, miR-148b#, miR-30d, and miR-589 (IFN- α /RBV treatment reduced plasma viral load by $0.80 (\pm 0.33) \log_{10}$ copies/ml during

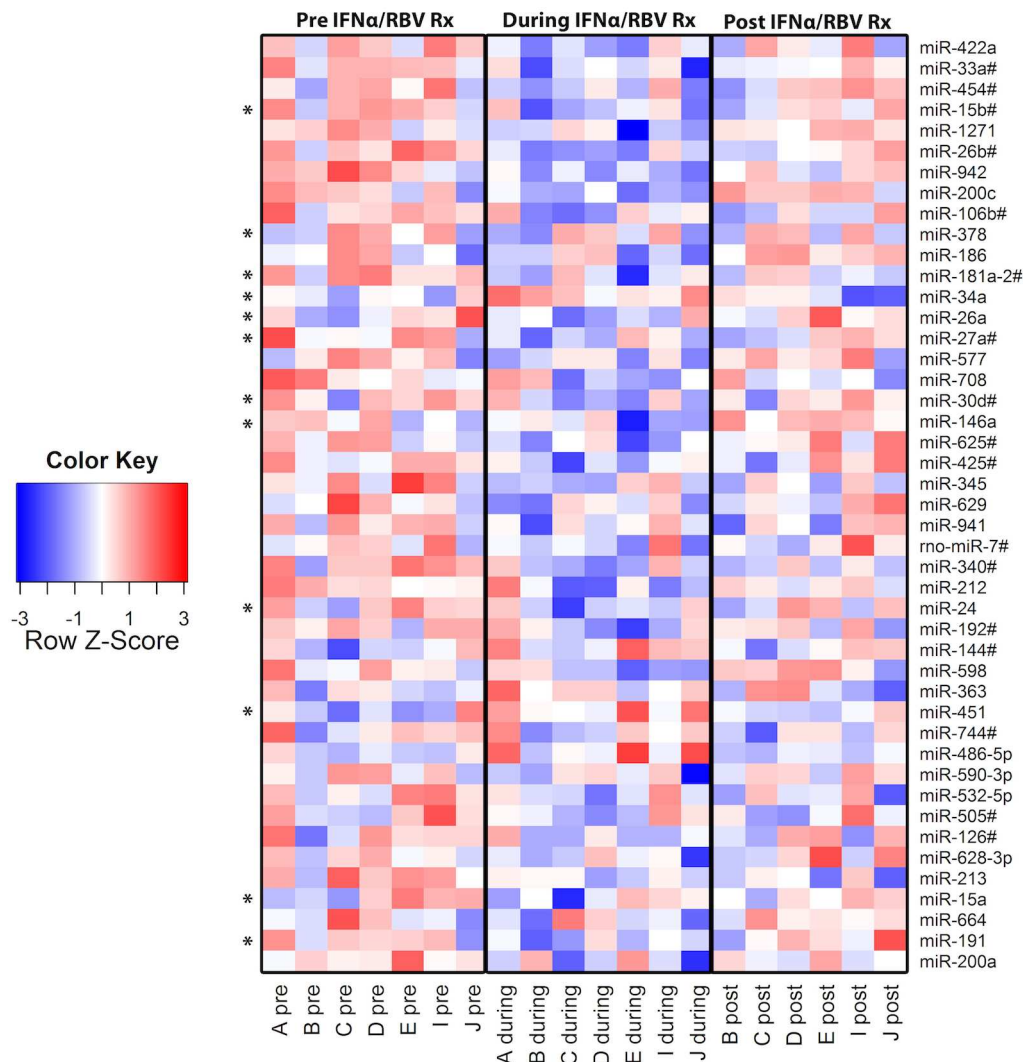


Figure 1. Heat map representing effects of IFN- α /RBV treatment on the expression of individual miRNAs. 45 miRNAs were significantly modulated by IFN- α /RBV *in vivo*, based on a paired t test and an uncorrected significance cutoff of $p < 0.05$. Relative copy numbers of each miRNA are reported. Blue color indicates ≤ -3 SD from the mean, red indicates ≥ 3 SD from the mean, and white represents the mean. Asterisks indicate previously established association with HIV-1.
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treatment, and viremia typically returned to approximate pre-treatment levels following therapy). Of particular relevance in these lists, miR-29a and miR-138 have been demonstrated to target HIV-1 directly [35], and both miRNAs demonstrated a negative correlation with HIV viral load in the current study.

Based on our data suggesting that miR-422a is the only miRNA that is significantly modulated by IFN- α *in vivo* in PBMCs, we performed a comprehensive search of the literature to identify established gene targets that are known to be regulated by miR-422a. To the best of our knowledge, there are only two experimentally verified regulatory targets of miR-422a: CYP7A1, a gene involved in regulation of bile acid synthesis in the liver [36], and MLH1, a gene involved in DNA mismatch repair that is a MutL α component [37]. According to a study by Mao et al [37], miR-422a negatively regulates the expression of MLH1. Overexpression of MLH1 induces apoptosis by blocking transcription on damaged DNA templates, resulting in p53 induction [38,39]. The IFN- α -mediated suppression of miR-422a observed in our preliminary experiments would be expected to result in increased

expression of MLH1, which in turn should elevate p53 expression, resulting in the induction of apoptosis. We sought to confirm these predicted relationships using our existing PBMC specimens from the seven SHCS patients, by measuring the mRNA expression of MLH1 and TP53 (the gene encoding p53) before and during IFN- α /RBV treatment. As predicted, both MLH1 and TP53 expression were elevated during the IFN- α /RBV treatment period (Fig. 2C and 2D), and a near perfect correlation was observed between the induction of the two genes (Fig. 2E). Although this does not represent direct evidence of a regulatory relationship between miR-422a and MLH1 (and TP53), the observed expression patterns are compatible with this regulatory scenario and consistent with the published literature.

We aimed to expand our understanding of the miR-422a interactome beyond direct experimentally-validated relationships using a combination of experimental and bioinformatic techniques. We hypothesized that IFN- α -modulated miRNAs suppress HIV-1 replication by enhancing the expression of anti-HIV-1 host restriction factors. We recently characterized the mRNA expres-

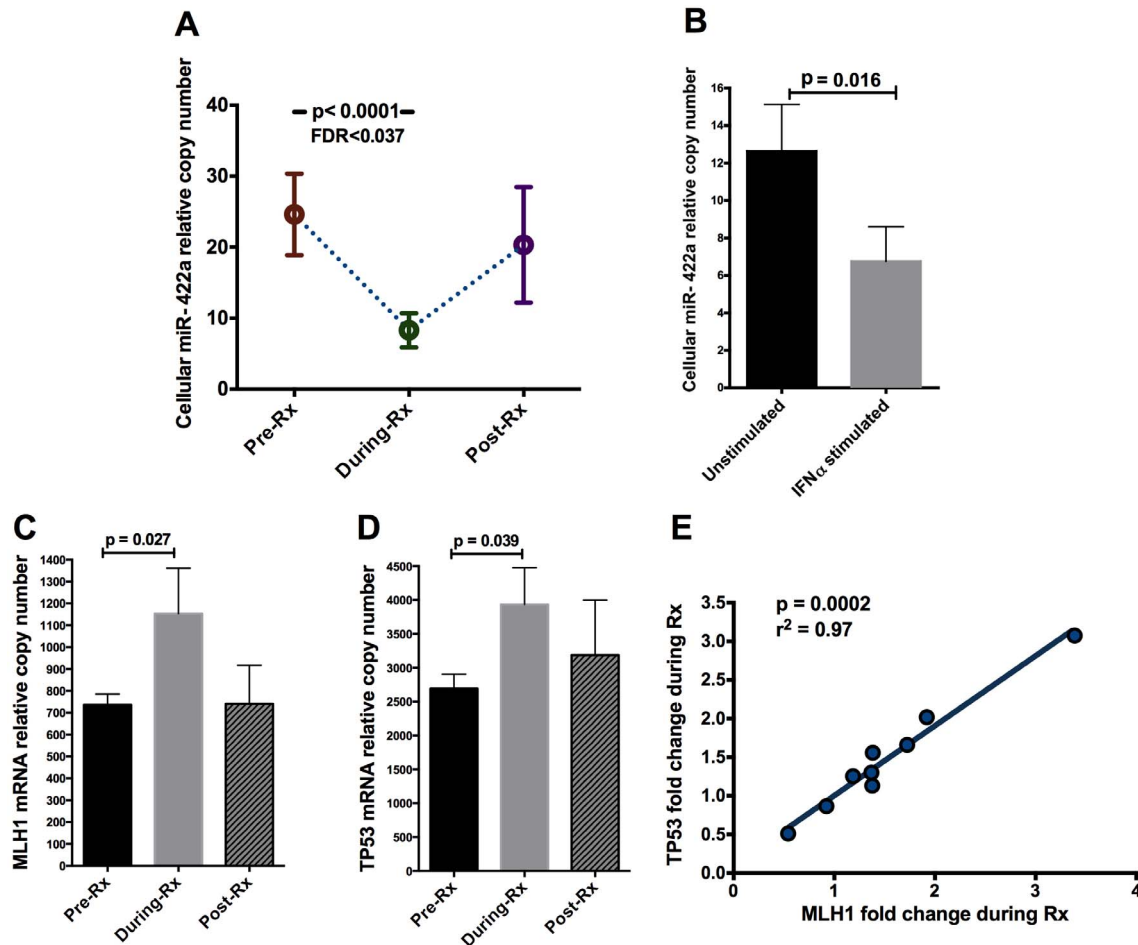


Figure 2. miR-422a is modulated by IFN- α treatment. (A) Expression of miR-422a in PBMC *in vivo* before, during and after IFN- α /RBV treatment (labeled as "Rx"). Error bars represent SEM. P-value was obtained using a paired t-test and FDR is reported. (B) Effects of IFN- α on the expression of miR-422a in CD4+ T cells *in vitro*; cells were plated at a million cells per well and treated with either 5 U/ml of IFN- α or media as a negative control. (C) Expression of MLH1 in PBMC *in vivo* before, during, and after IFN- α /RBV treatment. Error bars represent SEM. P-values were obtained using paired Wilcoxon tests. (D) Expression of TP53 in PBMC *in vivo* before, during, and after IFN- α /RBV treatment. Error bars represent SEM. P-values were obtained using paired Wilcoxon tests. (E) Correlation between TP53 fold induction and MLH1 fold induction in PBMC *in vivo* during IFN- α /RBV treatment. P-value was obtained using Spearman's rank test. doi:10.1371/journal.pone.0109220.g002

sion of all established anti-HIV-1 host restriction factors in our collection of longitudinal SHCS samples, demonstrating that IFN- α treatment significantly induces several restriction factors *in vivo* [15,16]. We exploited our previously generated gene expression data to simultaneously visualize IFN- α effects on miRNA and restriction factor mRNA profiles *in vivo*. Our visualization demonstrated that global miRNA expression was typically suppressed by IFN- α treatment, while restriction factors were typically induced (Fig. 3A). The distribution of data was highly and significantly skewed; miRNAs predominantly occupied the upper-left quadrant (representing suppression during IFN- α treatment and rebound to approximate baseline levels post-treatment), while restriction factor mRNAs predominantly occupied the lower-right quadrant (representing induction during IFN- α treatment and rebound to approximate baseline levels post-treatment). Although these correlative data do not prove that negative regulatory relationships exist between miRNAs and restriction factor mRNAs, the observed pattern is compatible, provocative, and worthy of further consideration.

We next implemented Ingenuity Pathway Analysis (IPA) software to perform integrative bioinformatic analyses of our miRNA, restriction factor, MLH1 and TP53 mRNA profiles

within the context of gene regulatory networks. By merging our expression data from SHCS subjects with the Ingenuity Knowledge Base, IPA assembled a rich genetic interaction network depicting known experimentally validated relationships (Fig. 3B). There are a few prominent features within the IPA network that warrant mention. First, in addition to the previously known relationship with MLH1, miR-422a is predicted to directly regulate TP53 and the tripartite motif (TRIM) family anti-HIV-1 restriction factors TRIM19 (PML) and TRIM22. This prediction is based on sequence homology between the miR-422a seed region sequence and the 3' UTR of TP53, TRIM19 (PML) and TRIM22. In alignment with this prediction, both TRIM19 (PML) and TRIM22 are induced by IFN- α /RBV according to our published data [16]. In addition to putative antiviral functions, TRIM19 (PML) is a potent driver of DNA damage-induced apoptosis, and physically interacts with p53 *in vitro* and *in vivo*. PML acts as a transcriptional co-activator with p53 and potentiates the antiproliferative downstream effects of p53 [40]. Second, TP53 is the epicenter of the miR-422a genetic interaction network, exhibiting numerous direct and indirect connections with host restriction factors. Accordingly, IFN- α has been shown to enhance the transcription of p53 target

Table 1. Correlations between miRNA and HIV-1 viral load.

microRNA	Spearman r	p value	R square	Previously associated with HIV? Y/N [ref]
Correlations between pre-IFN-α/RBV miRNA relative copy number and HIV-1 viral load.				
miR-25#	0.919	0.007	0.393	N
miR-885-5p	0.857	0.024	0.888	N
miR-195	-0.821	0.034	0.426	Y [55]
miR-29a	-0.786	0.048	0.635	Y [56,57]
miR-101	-0.786	0.048	0.244	Y [58]
miR-503	-0.786	0.048	0.210	Y [59]
miR-491-5p	-0.786	0.048	0.191	N
Correlations between miRNA relative copy number and HIV-1 viral load during IFN-α/RBV.				
miR-145#	0.883	0.015	0.696	N
miR-138	-0.857	0.024	0.746	Y [35]
miR-10a	-0.821	0.034	0.406	N
miR-31	-0.786	0.048	0.755	Y [59,60]
miR-589	-0.786	0.048	0.536	N
let-7e	-0.786	0.048	0.507	Y [61]
Correlations between miRNA expression fold change and HIV-1 log viral load reduction.				
miR-30e-3p	0.821	0.034	0.522	Y [62]
miR-148b#	0.786	0.048	0.547	Y [63]
miR-30d	0.786	0.048	0.488	Y [55]
miR-589	0.786	0.048	0.431	N

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genes and p53-dependent apoptosis, and can directly induce expression of p53 [41]. Lastly, the gamma interferon-inducible gene IFI16 appears in the network. IFI16 has recently been characterized as a DNA sensor that plays a critical role in triggering the caspase 1-mediated pyroptosis of abortively-infected cells [42], which may be a principal mechanism underlying CD4 T cell depletion in HIV-1-infected individuals [43]. Taken together, the IFN- α -suppressed miRNA miR-422a is embedded

in a rich interactome associated with both control of viral replication and apoptotic induction.

To complement our focused analyses of miR-422a, we developed and implemented a computational approach to infer regulatory networks between the entire repertoire of surveyed miRNAs and the mRNA expression of anti-HIV-1 restriction factors observed in our IFN- α /RBV-treated SHCS subjects. Our approach is derived from a similar experiment used to examine miRNA-mRNA pairs within the context of HCV infection [44].

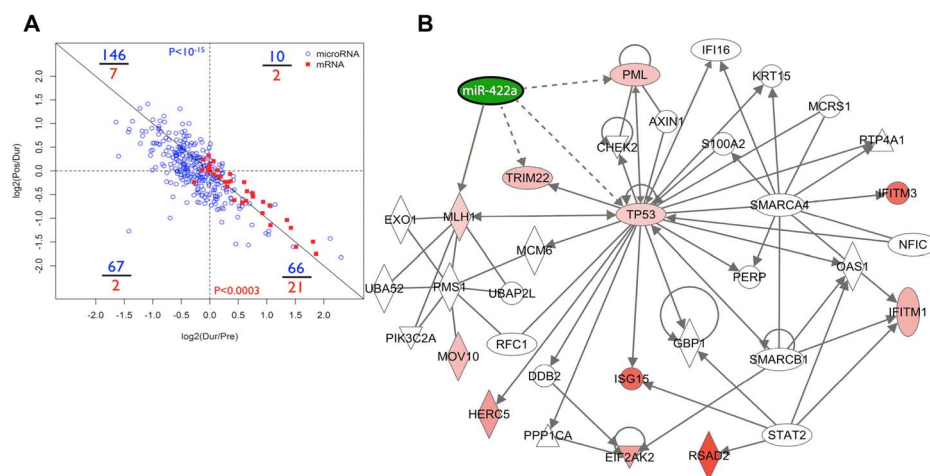


Figure 3. Visualization of miRNA and mRNA regulatory networks. (A) Plot of global miRNA and anti-HIV-1 restriction factor responses to IFN- α /RBV treatment. Numbers in blue and red represent tallies of microRNAs and restriction factor mRNAs in each quadrant, respectively. P-values were obtained using Fisher's exact tests. (B) Integrative bioinformatic analyses of our miRNA data, restriction factor, MLH1 and TP53 mRNA profiles within the context of gene regulatory networks. Ingenuity Pathway Analysis (IPA) software was implemented to create the network map.

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We used two variables to generate the network: 1) inverse expression relationships between a given miRNA-mRNA pair, and 2) significant sequence homology between a given miRNA seed region and a restriction factor 3' UTR (word size 4, alignment length ≥ 5 , e-value < 1.0). These relationships were interpreted as putative negative regulatory interactions between a miRNA and mRNA. Our approach revealed a large number of potential regulatory interactions between miRNAs and restriction factor mRNAs (Fig. 4); 15 out of the 34 restriction factors that were up-modulated in SHCS patients undergoing IFN- α /RBV treatment were associated with at least 1 putative regulatory miRNA, and 29 distinct miRNAs were involved in these predicted relationships. To evaluate the likelihood that our network inference strategy was revealing legitimate regulatory relationships, we compared the frequencies of significant miRNA seed sequence - mRNA 3' UTR homology hits between miRNA - mRNA pairs with significant inverse expression relationships (35 out of 62) and pairs without inverse expression correlations (8455 out of 25,636). Using a Fisher's Exact test, we were able to determine that miRNA-mRNA sequence homology was observed at a significantly higher frequency in inverse expression relationships ($p = 8 \times 10^{-6}$, OR = 3.2 [95% CI 1.9, 5.6]), implying that the identified miRNA-mRNA networks might in fact play a role in regulating restriction factor expression.

Our analyses revealed a provocative list of 22 distinct potential miRNA regulators of TRIM21. The TRIM family contains over 60 proteins and exhibits a wide range of activities, including viral suppression and regulation of innate and adaptive immune responses. TRIM21 is known to play a crucial role in regulating type I interferon production [45]. Recently, TRIM21 was

reported to recognize and degrade viruses in the cytoplasm by binding to antibody-coated virions [46–48]. Therefore, TRIM21 acts as an intracellular arm of adaptive immunity, and serves as a direct link between cell-intrinsic and adaptive immune processes. Our list of predicted miRNA regulators of TRIM21 expression warrants further investigation and may be exploited to enhance the potent antiviral activity of TRIM21 *in vivo*.

Our collection of clinical samples does not allow us to evaluate the possibility that the inclusion of ribavirin in anti-HCV therapy and universal HCV-coinfection in our cohort may affect our findings. However, ribavirin treatment has been previously shown to have negligible effects against HIV-1 (56). Moreover, gene expression profiles of HIV-1-monoinfected individuals undergoing IFN- α monotherapy [5,49] match our gene expression data from IFN- α /RBV-treated HIV/HCV-coinfected patients [15,16]. We confirmed the IFN- α -mediated suppression of miR-422a by performing an *in vitro* experiment in isolated peripheral blood CD4+ T cells in the absence of ribavirin and HCV infection, which extends our *in vivo* observations in IFN- α /RBV-treated HIV/HCV-coinfected individuals. Although our principal result involving miR-422a is not likely to have been affected by ribavirin administration or HCV coinfection, future miRNA profiling studies of HIV-1-monoinfected individuals undergoing IFN- α monotherapy will complement our observations reported here.

Amongst the HIV/HCV-coinfected participants enrolled in the Swiss HIV Cohort Study, ~85% report intravenous drug use. In regards to potential confounding effects of IDU status on our findings, morphine usage may affect HIV disease outcomes and accelerate disease progression [50,51]. Moreover, *in vitro* studies have demonstrated that morphine treatment increases cellular

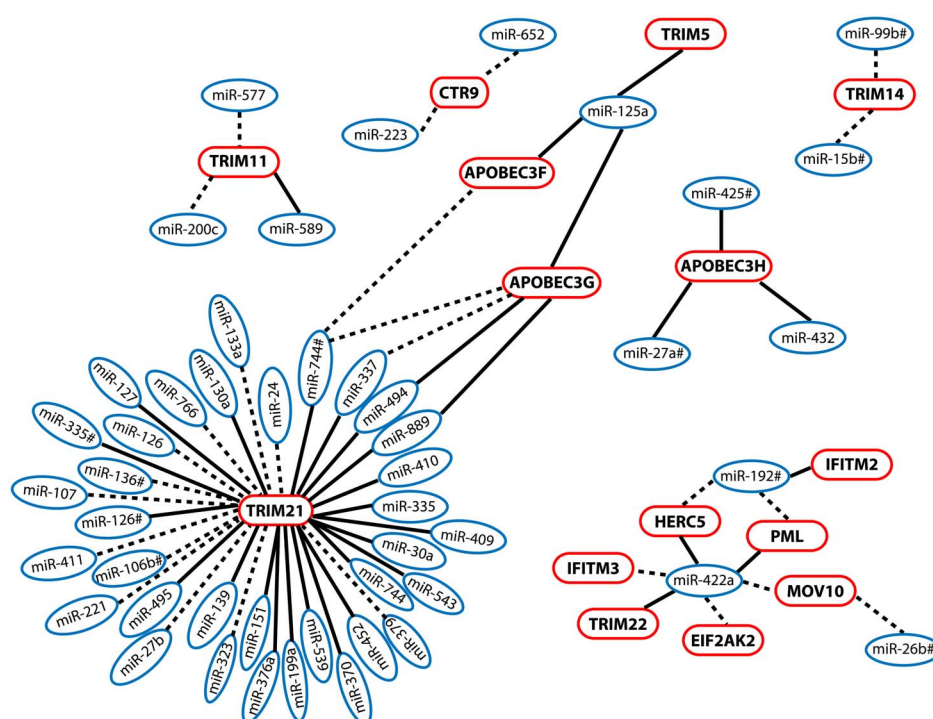


Figure 4. Global network of putative regulatory interactions between miRNAs and anti-HIV-1 restriction factor mRNAs. miRNA and mRNA expression data were measured in longitudinal PBMC samples from IFN- α /RBV-treated patients enrolled in the SHCS. Two variables were used to generate the network: 1) inverse expression relationships between a given miRNA-mRNA pair (represented as a dashed line drawn between a miRNA – mRNA pair), and 2) Significant sequence homology between a given miRNA seed region and a restriction factor 3' UTR (word size 4, alignment length $>= 5$, e-value < 1.0). Significant sequence homology is represented by a solid line. miRNAs are listed in blue; restriction factors are listed in pink.

susceptibility to HIV infection, inhibits production of IFN- α and IFN- β antiviral cytokines [52], and modulates the expression of anti-HIV-1 miRNAs (miRNA-28, 125b, 150, and 382) [53]. Importantly, none of these opioid-modulated miRNAs were modulated in response to IFN- α /RBV therapy in our study.

In summary, our data demonstrate that a specific reduction of cellular miR-422a is associated with the suppression of HIV-1 by IFN- α *in vivo*. Our tiered network analyses suggest that miR-422a may contribute to the IFN- α -mediated suppression of HIV-1 viremia and decay of the latent reservoir via regulation of multiple retroviral restriction factors and genes involved in p53-dependent apoptosis and pyroptosis pathways. In addition, our global miRNA surveys identified several miRNAs whose expression levels were significantly correlated with HIV-1 viral load, and 29 distinct miRNAs that may regulate anti-HIV-1 restriction factor expression *in vivo*. Comprehensive miRNA profiling of isolated cellular subsets (e.g. CD4+ T cells, CD8+ T cells, B cells and monocytes) in subsequent studies will complement these observations. The possibility exists that one or more of these identified miRNAs may be manipulated to control HIV-1. *In vivo* targeting of the liver-specific miRNA and essential HCV cofactor miR-122 by the small molecule drug “miravirsin” achieves up to a three-log reduction in HCV viral load which persists indefinitely after treatment cessation [54]. This provides a promising model, and suggests that the development and deployment of miRNA-based therapeutic strategies for other chronic viral pathogens including HIV-1 is likely achievable.

To the best of our knowledge, our study is the first to demonstrate the effect of IFN- α treatment on miRNA expression profile *in vivo*, and these are the first data associating miR-422a with HIV-1 infection. Future work should validate and extend the translational and *in silico* observations reported here with detailed *in vitro* analyses of miR-422a effects on HIV-1 replication and the lifespan of HIV-1-infected cells.

Supporting Information

Figure S1 IFN- α /RBV treatment does not alter the expression of microRNA machinery genes *in vivo*.

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Expression of (A) DROSHA, (B) DICER1, and (C) DGCR8 miRNA machinery genes before, during, and after IFN- α /RBV treatment. Black, light grey, and dark grey bars represent pre-treatment, during treatment, and post-treatment expression levels, respectively. P-values were obtained using paired Wilcoxon tests. (PDF)

Table S1 SHCS subject characteristics at baseline (pre IFN- α /RBV treatment).

(PDF)

Table S2 List of microRNAs modulated by IFN- α /RBV with p-value<0.05 (pre Rx/during Rx).

(PDF)

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Conceived and designed the experiments: MAM AR BL PJN HFG JKW SKP. Performed the experiments: MAM AD TL EJ SKP. Analyzed the data: MAM XD SKP. Contributed to the writing of the manuscript: MAM SKP.

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